Bangladesh J. Bot. **44**(5): 727-736, 2015 (December), Supplementary

GENETIC DIVERSITY OF WILD *CONYZA BLINII* H.LÉV. BASED ON RAPD AND SRAP MARKERS

SHAN LIU, RONG SUN[1] AND HUI CHEN[1]

Department of Biological and Chemical Engineering, Panzhihua University, 617000, Panzhihua, China

Key words: *Conyza blinii,* RAPD, SRAP, Genetic diversity, HPLC

Abstract

The genetic diversity and genetic relationships among 16 populations of *Conyza blinii* in the Panxi area of China were assessed by RAPD and SRAP markers. Relative to independent molecular markers, the combined RAPD-SRAP data showed a more reasonable cluster (r = 0.80718), and the clustering results divided the 16 populations into three groups. One major group was formed by populations from Pzhihua city (nos. 1 to 7) and another two populations from Liangshan prefecture (nos. 8 and 9); two populations (nos. 10 and 11) collected from Liangshan clustered into the second category, and the remaining populations made up cluster III. The samples clustered into two groups based on the content of blinin (the characteristic compound of this plant), except samples 4, 5 and 16, other samples were clustered similar like molecular markers, they all gathered together based on their similar distance. The Jaccard's similarity coefficients ranged from 0.5073 to 0.8422, the results suggested that the genetic variation of wild populations was not abundant. However, based on blinin content (detected by HPLC), there was a large difference among populations although populations had a close genetic relationship which revealed that samples were not fully related to their geographical location. The result has important implications for *C. blinii* characterization, improvement, and conservation.

Introduction

Conyza blinii H. Lév. is a traditional Chinese medicinal plant. The wild type has only been reported in the Southwest China. The two years old *Conyza blinii* H. Lév. (family Compositae) is distributed mainly in the Yunnan and Sichuan provinces of China. It is most abundant in the Panxi area of Sichuan with its arid, hot weather, high altitude and abundant sunlight, an environment that is best suited to *C. blinii* growth (Committee 2010). The Panxi area, located in southwestern Sichuan, is part of the upper Yangtze River region, which comprises Panzhihua city and Liangshan prefecture. It consists of 67,549 km^2 of land area, and forms a unique light and heat climatic zone due to the influence of the north-south flow of the wind and its unique topography. The Panxi area has become famous for the production of *C. blinii*, with annual herb sales accounting for a sizeable portion of the economy in Sichuan province (Xu *et al.* 2004).

This herb was developed as a folk medicine in the 1970s because of its excellent curative effect on chronic bronchitis (Committee 2010). Some reports have also indicated that *C. blinii* extract can be used to treat gastric ulcers (Su *et al.* 2007) due to its unique chemical composition of blinin (Yang *et al.* 1989). In recent years, Chinese researchers have been studying on cultivation management of *C. blinii* to elucidate its chemical constituents. Unfortunately work on its identification and evaluation at the molecular level is meagre.

Genetic diversity of this species have been undertaken using different molecular markers. However, use of a single marker technique to study genetic diversity may lead to erroneous and

[1]Author for correspondence: <htsof@163.com>. [1]Department of Biology and Science, Sichuan Agricultural University, 625014,Ya'an, China.

unreliable results. This is perhaps the limitations of molecular-marker technology. Therefore, in this study random-amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers were used to analyze genetic diversity of *C. blinii*, as has been done for *Brassica campestris* L. ssp. chinensis (Ma *et al.* 2008), *Pistacia vera* (Wang *et al.* 2012), *Salvia miltiorrhiza* (Song *et al.* 2009), *Saccharum spontaneum* (Chang *et al.* 2012), kenaf (Xu *et al.* 2013), and *Chrysanthemum morifolium* (Shao *et al.* 2010) and *Cucumis sativus* L. (Manohar *et al.* 2013), among others.

Still *C. blinii* only grows as wild, as a result cannot satisfy market demand. The aim of the present study was to use RAPD and SRAP makers to determine the magnitude of the genetic diversity and the genetic relationships among 16 populations of *C. blinii* for future marker assisted genetic breeding-programme.

Materials and Methods

Sixteen wild populations of *C. blinii* were sampled from the Panxi area (Fig. 1, Table 1). Five plants were randomly collected from each population, at a minimum interval of 10 m to avoid analytical errors caused by individual differences. Total genomic DNA was extracted from young leaves dried in silica gel in the field, using a modified SDS method (Liu *et al.* 2013). DNA quality and quantity were checked in 1% (w/v) agarose gels and with a Unico SmartSpec^{TM} Plus Spectrophotometer (Bio-Rad, USA).

Table 1 Geographical information for the 16 populations of *Conyza blinii.*

No.	Location	Altitude (m)	Habitat	No.	Location	Altitude (m)	Habitat
1	Salian, Panzhihua	1800	Slope shrub	9	Xinyun, Liangshan	2000	Roadside
2	Lisu, Panzhihua	2200	Slope shrub	10	Ninnan, Liangshan	2000	Slope shrub
3	Tounian, Panzhihua	2012	Slope shrub	11	Xintian, Liangshan	1800	Roadside
4	Xinjiu, Panzhihua	2100	Roadside	12	Butuo, Liangshan	2200	Slope shrub
5	Zhongfa, Panzhihua	1500	Riverside	13	Dechang, Liangshan	2100	,,
6	Lazha, Panzhihua	1600	Slope shrub	14	Xichang, Liangshan	1900	,,
7	Taiping, Panzhihua	1800	Slope shrub	15	Yanyuan, Liangshan	1800	,,
8	Longquan, Liangshan	2400	Slope shrub	16	Mianning, Liangshan	1200	Artificial culture

Fifteen selected 10-mer random primers (Table 2) were used for PCR amplification (Life Technologies, Shanghai). RAPD-amplification reactions were carried out using pre-optimized methods in a 25-μL reaction volume containing 2.5 mM Mg^{2+}, 4.5 mM dNTPs, 3.5 μM primers, 1.5 U Taq DNA polymerase (TaKaRa Taq^{TM}, Shanghai, China), and 60 ng genomic DNA template. PCR amplification was performed in a Bio-Rad S1000 Thermal Cycler under the following conditions: 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, and a final extension of 10 min at 72°C. Amplified products were analyzed on 1.8% (w/v) agarose gels containing 1% (v/v) GoldView (Solarbio Company).

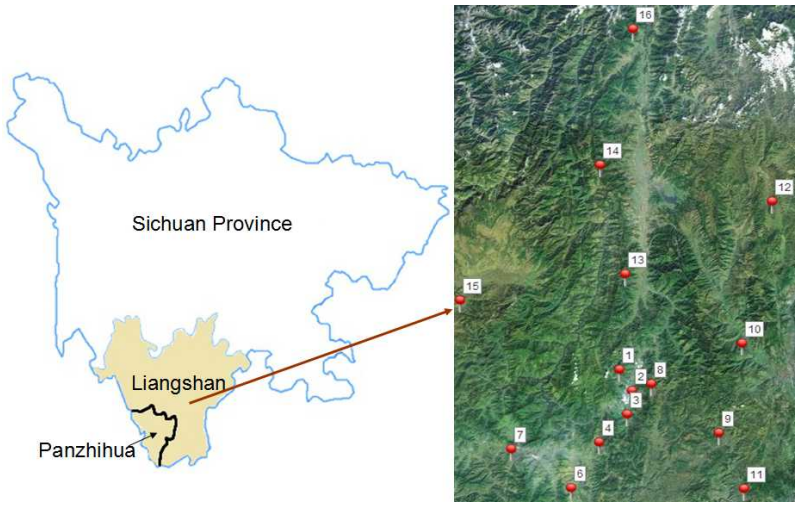

Fig. 1. Map showing the areas from which the 16 *Conyza blini* populations were collected. Population codes are as specified in Table 1.

The SRAP-PCR primers reported by Li and Quiros (Li and Quiros 2001) were used in this study, and 12 primer pairs (Life Technologies, Shanghai; Table 3) which clearly amplified bands were selected for the genetic-diversity analysis. Each 15-µl reaction contained 0.15 mM dNTPs, 50 ng DNA template, 0.7 µM primers, 0.7 U Taq DNA polymerase (idem as before) , 1.9 mM Mg^{2+} and 2-µl 10× PCR buffer. The PCR program was: 5 min of initial denaturation at 94°C, 5 cycles of 1 min denaturation at 94°C, 1 min annealing at 35°C, and 1 min extension at 72°C; then, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 51.3°C, and 1 min extension at 72°C, with a final extension of 10 min at 72°C. Amplified products were electrophoresed on an 8% (w/v) non-denaturing polyacrylamide gel and detected by silver-staining followed by allele scoring for better accuracy.

Table 2. List of forward and reverse RAPD primers used in this study.

Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)
OPO-5	CCCAGTCACT	UBC356	GCGGCCCTCT
OPO-20	ACACACGCTG	UBC362	CCCAAGGTCC
H13	ACCCGACACT	S10	CTGCTGGGAC
H19	GAGTCAGCAG	S33	CAGCACCCAC
H29	CCGCATCTAC	S45	TGAGCGGACA
H35	TGCGCTCCTC	S266	AGGCCCGATG
H37	AGCGCCATTG	S2033	CTTCGGTGTG
H38	ACCCGGTCAC		

Table 3. List of forward and reverse SRAP primers used in this study.

Primer	Forward primer (5'→3')	Reverse primer (5'→3')
me1+em1	TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAT
me1+em3	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGAC
me1+em6	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGCA
me2+em1	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAT
me2+em3	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTGAC
me2+em4	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGA
me2+em5	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAC
me3+em2	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTTGC
me3+em4	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTTGA
me4+em1	TGAGTCCAAACCGGACC	GACTGCGTACGAATTAAT
me4+em4	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTGA
me4+em6	TGAGTCCAAACCGGACC	GACTGCGTACGAATTGCA

The *C. blinii* materials collected from 16 populations were cleaned and stored at room temperature under light-protected and humidity-proof conditions until ground for chemical analysis. For the determination of dry mass, the samples were kept in the oven at 50°C for drying to constant weight (Peng *et al.* 2011). The active component (Blinin) was macerated with methanol and extracted by the Soxhlet method: 0.20 g of dried and powdered sample was placed on a filter paper in the thimble holder of the Soxhlet apparatus and extracted with 20 ml MeOH at 65°C until the liquid turn to colorless in a water bath. Then, liquid was treated with 150 ml of chloroform ($CHCl_3$) to remove the chlorophyll contents. The extract was then filtered and diluted to 25 ml volume using methanol.

A reversed-phase C18 column (Agilent Eclips XDB, 4.6 mm×150 mm) was used with the mobile phase consisted of the eluent acetonitrile : methanol : water as 15 : 40 : 45. The column compartment was kept at the temperature of 25°C and the sample volume injected was 20 μl. Peaks were detected at 210 nm.

Blinin is the typical medicinal components of *C. blinii*, so it was considered as the reference substances in fingerprint. Stock solution of blinin (120 μg/ml) was prepared by dissolving suitable amount of pure blinin in methanol. After filtered, the reference substance solution was directly injected into the HPLC. The standard curve was obtained by plotting the peak areas of standard concentrations of blinin (7.2 - 120 μg/ml). The concentration of flavonoids was expressed as μg/g dry matter (DM). Values represent the mean of analysis of independently extracted samples.

In the final PCR results, only bands that could be unambiguously scored across all of the sampled populations were used in this study. The RAPD- and SRAP-amplified bands were scored as "1" for presence and "0" for absence of polymorphic bands for all samples. The resulting data matrix was analyzed using the PopGen32 computer program (Rohlf 1998) to estimate six parameters of genetic diversity - percentage of polymorphic bands (PPB), Shannon's information index (I), the observed number of alleles (N_a), the effective number of alleles (N_e), Nei's measure of genetic diversity (H) (Nei 1972), and the coefficient of genetic distance (GD), equal to 1 minus the genetic similarity (GS) (Nei 1973) - among the groups of 16 populations. The dendrogram was constructed by unweighted pair group method with arithmetic average (UPGMA) using the SAHN

module of NTSYSpc 2.20 software. The programs COPH and MXCOMP were used to calculate goodness-of-fit between the cluster analysis and the original matrix from each marker, and the similarity between RAPD and SRAP matrices.

All extractions and determinations were performed with at least three replications to allow statistical analysis. The one way analysis of variance (ANOVA) with SPSS 20.0 was used to assess differences between the 16 populations. The cluster analysis (CA) was applied to compare and group accessions according to content of secondary metabolites.

Results and Discussion

The 15 selected RAPD primers generated 147 amplified products with band sizes ranging from 200 to 2,000 bp. Each primer amplified from 4 (H35) to 12 (OPO-20) polymorphic bands, with on an average 7 polymorphic bands per primer. Of these bands, 140 showed polymorphism with a PPB of 97.14%. The GS of the RAPD amplification results ranged from 0.4789 to 0.9014, with an average of 0.6732. Jaccard's GS for the RAPD markers revealed maximum GD (0.5633) between the samples from Dechang and Xichang, and minimum GD (0.0704) between the samples from Lisu and Ninnan. This indicated that the latter pair had the highest GS and the former pair, the lowest. The matrix comparison plot test (Fig. 2 a) showed a high goodness-of-fit for the cluster tree on the original distance matrix (r = 0.85837). The populations separated into four main clusters with a genetic similarity of 0.676 (Figs 2a, 3a): cluster I contained populations 1 to 10 (Table 1 for population numbers), and it included all of the samples from Panzhihua city and three samples (8, 9 and 10) from Liangshan, which is geographically close. Cluster II contained only sample 11. Cluster III included samples 12, 13 and 16, all from the state of Liangshan, but from a geographical point of view, sample 16 was furthest from the other samples. Samples 14 and 15, both from Liangshan, made up cluster IV.

Populations for which the SRAP primer combinations gave high polymorphism and strong-signal bands were selected. The total number of bands amplified at the species level was 341, ranging from 7 (me2 + em1) to 25 (me3 + em4), 318 of which were polymorphic with a PPB of 92.38%. The GS of the SRAP amplification results ranged from 0.4956 to 0.8797, with an average of 0.6804. The data from the SRAP markers revealed maximum GD (0.504), and thus lowest GS, between the samples from Longquan and Xinyun on contrast the minimum GD (0.129), and thus highest GS, between samples from Longquan and Xichang. Matrix comparison plot test (Fig. 2 b) showed a high goodness-of-fit for the cluster tree on the original distance matrix (r = 0.74746). The populations separated into three main clusters with a coefficient of 0.699 (Figs 2b, 3b): cluster I contained populations 1 to 5 and 7 to 9; it included most samples from Panzhihua city, except sample 6, and samples 8 and 9 from Liangshan prefecture. These latter samples probably clustered in this category because in terms of geographical distance, closer to Panzhihua than the other Liangshan populations. Samples 10 and 11 made up cluster II. Cluster III included samples 12 to 16, all from the state of Liangshan. Unexpectedly, sample 6 from Panzhihua also clustered in this category, although from a geographical point of view it seemed more likely to be assigned to cluster I.

Tables 4 and 5 show Nei's unbiased measures of GS among the 16 populations of *C. blinii.* The total genetic tendency of the RAPD and SRAP data was similar.

Table 4. Genetic diversity of the 16 populations by RAPD.

	na	Ne	H	I
Mean	1.9296	1.5684	0.3231	0.4804
Sd	0.2577	0.3650	0.1736	0.2250

Table 5. Genetic diversity of the 16 populations by SRAP.

	na	Ne	H	I
Mean	1.9238	1.4192	0.2578	0.4013
St. Dev	0.2658	0.3296	0.1648	0.2180

na, observed number of alleles; Ne, effective number of alleles (Kimura and Crow 1964); H, Nei's (1973) measure of genetic diversity; I, Shannon's information index (Lewontin 1972).

Despite the very close genetic-relationship tendencies shown by the two markers, Mantel test showed that the GD values were not highly correlated between the RAPD and SRAP matrix data (r = 0.58442). To obtain more accurate genetic estimates, a combined analysis was carried out using all of the RAPD and SRAP data together. The GS of the combined RAPD-SRAP amplification results ranged from 0.5073 to 0.8422 with an average of 0.6786 while the GD ranged from 0.1578 (Longquan and Xinyun) to 0.4927 (Longquan and Xichang). The combined analysis showed that the genetic variation of wild populations was not abundant. This matrix, based on the combined data, was used to generate a dendrogram showing genetic relationships among the accessions. The populations separated into three main clusters with a coefficient of 0.684 (Figs 2c, 3c): cluster I contained populations 1 to 9, and included all of the samples from Panzhihua city (1 to 7) and two samples from Liangshan (8 and 9); samples 10 and 11 were found in cluster II; the remaining samples from Liangshan clustered into group III. This clustering result was not identical to that obtained using the RAPD and SRAP markers separately, in particular for the samples from the border area between Liangshan and Panzhihua. Matrix comparison plot test (Fig. 2. c) showed a high goodness-of-fit for the cluster tree on the original distance matrix (r = 0.80718), indicating a significant positive correlation between the two matrices. Thus the merged data were more stable and useful for interpretation of the genetic relationships among populations.

Analysis of genetic diversity showed significant similarity in the number of alleles. Nei's genetic diversity index and Shannon's information index (Tables 4 and 5), with the RAPD markers showing slightly higher values than the SRAP markers for these parameters. RAPD markers also showed higher PPB (97.14%) than the SRAP markers (92.38%). Clustering results showed that the genetic relationships among wild *C. blinii* populations were highly correlated with geographical location in the Panxi area (Figs 2a, 3a). In accordance with the division of origin, the SRAP results were more representative. However, the clustering results of each of the markers alone resulted in mixed classification of samples from different regions, for example, the clustering results of sample 10 and 11 are not the same.

A comparison of the data from RAPD, SRAP and combined RAPD - SRAP analyses revealed a matrix correlation for RAPD and RAPD - SRAP of 0.7460, and for SRAP and RAPD - SRAP of 0.8751. Both reflected a high degree of matching. The combined data were clearly more suitable for the identification and classification of genetic relationships. The combined RAPD - SRAP marker data seemed to be more similar to the SRAP results, but the matrix correlation for the former was higher (r = 0.80718) than the latter. Using the combined marker data, the 16 natural wild populations collected from the Panxi region clustered into three categories (Figs 2c and 3c). Genetic distance and geographical distance of wild *C. blinii* were associated, for example: cluster I contained all of the Panzhihua samples, as well as samples 8 and 9 from Liangshan (Figs 2c and 3c), even though simply based on location, they seemed more likely to belong to Panzhihua city (Fig. 1).

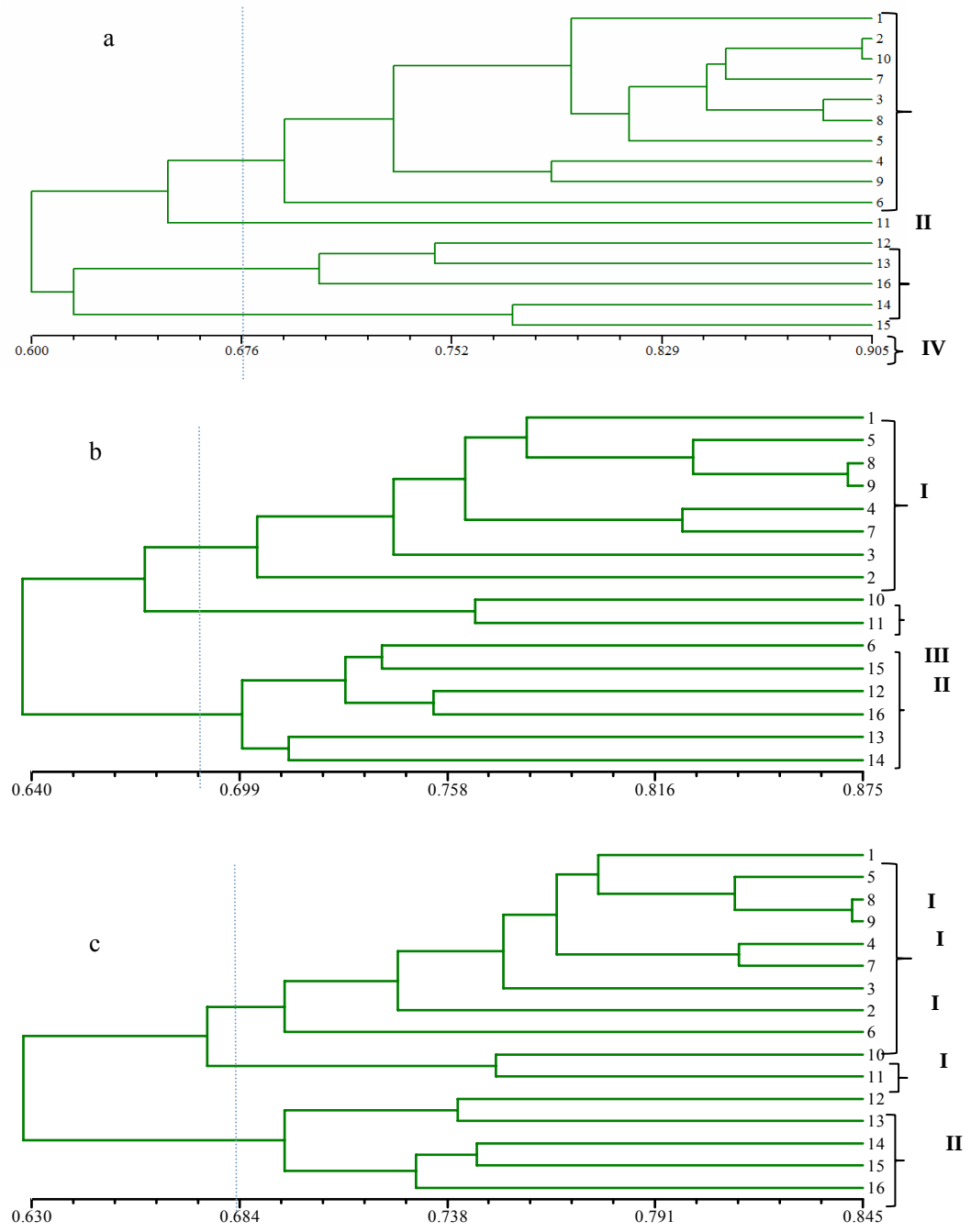

Fig. 2. UPGMA dendrogram of the genetic relationships based on (a) RAPD, (b) SRAP and (c) combined RAPD – SRAP analysis.

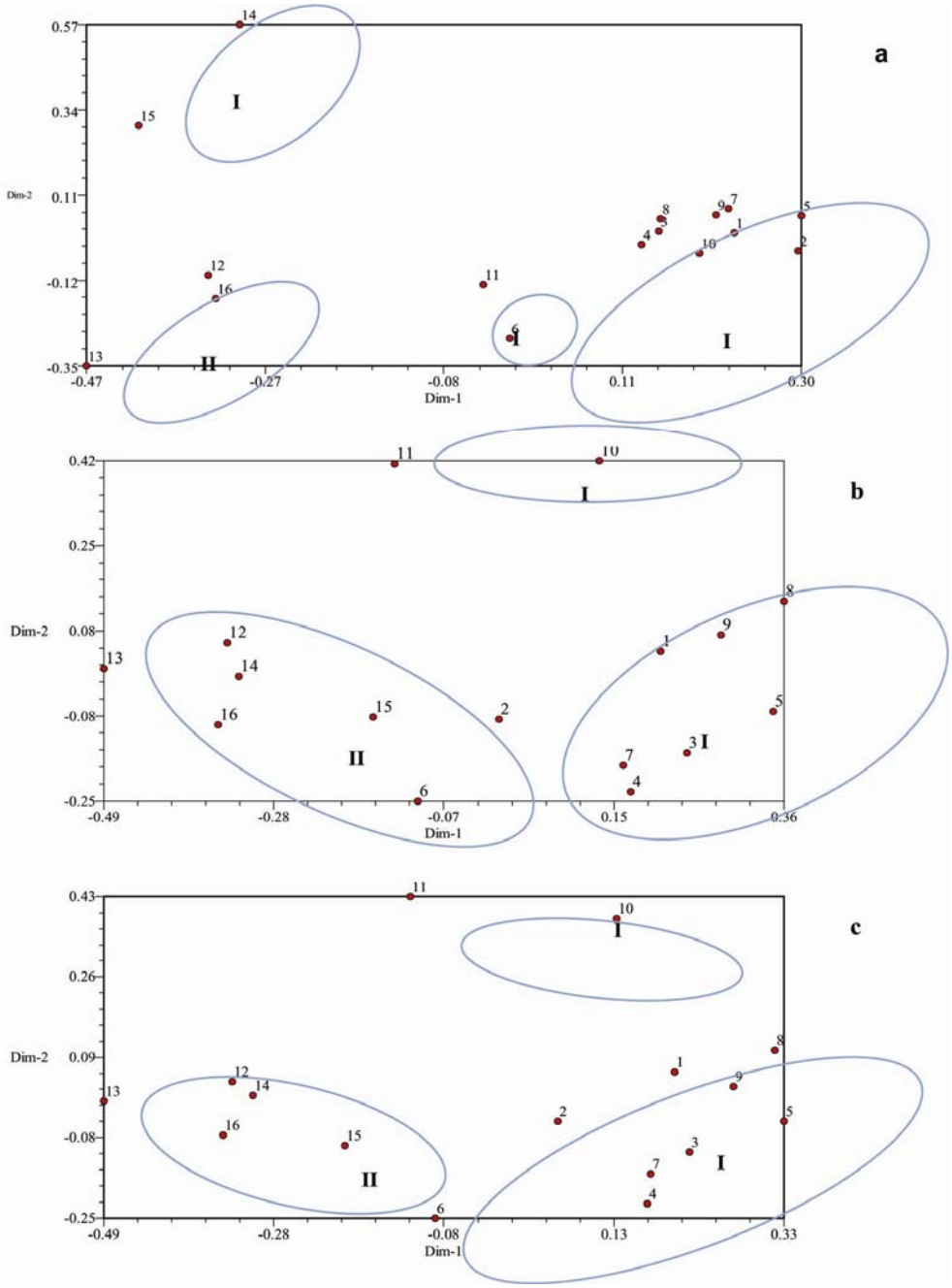

Fig. 3. Two-dimensional plot (with vectors) of principal coordinate analysis of *Conyza blinii* by (a) RAPD, (b) SRAP and (c) combined RAPD - SRAP analysis.

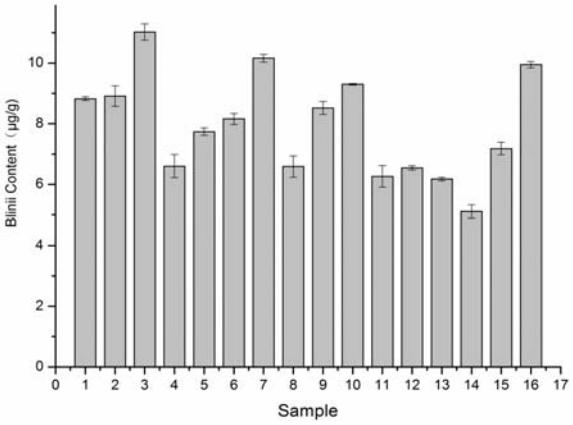

Fig. 4. Assay results of blinin content in different region of mature *Conyza blinii*.

Blinin is a neoclerodane diterpene existing only in *C. blinii.* The content of this feature compound employed to perform the cluster analysis among samples had a certain significance (Fig. 5). The result of the blinin content was given in Fig. 4. The amount were from 0.56% to 1.19%. The general structure of the dendrogram confirms the existence of two main clusters as the threshold of T = 15. The cluster I, included the accessions of 1, 2, 3, 6, 7, 9, 10, 16 while the cluster II included samples of 4, 5, 8, 11, 12, 13, 14, 15. This result showed a different clustering form between molecular markers. The most obvious was sample 10 and 11. In RAPD, SRAP and

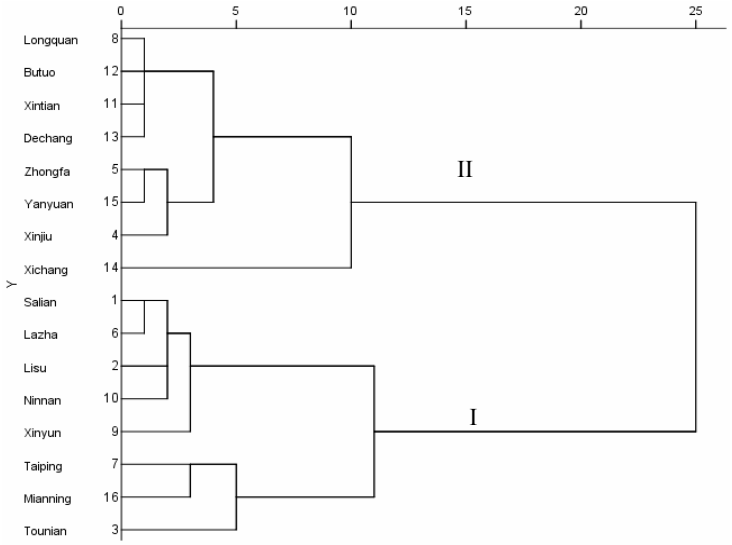

Fig. 5. The hierarchical cluster analysis dendrogram of *C. blinii* accessions made by the contents of blinii.

combined RAPD - SRAP analysis the both clustered together, (Fig. 2) but in the blinin content they varied greatly (Fig. 5). Except samples 4, 5 and 16 and other samples were clustered like those performed with the molecular markers, they all gathered together based on their similar distance.

Acknowledgements

This work was funded by the Sichuan Science and Technology Innovation Seedling Project Foundation (No. 2012ZZ046), and the program of Key Laboratory of Dry-Hot Valley Characteristic Bio-Resources Development at the University of Sichuan Province (No. GR-2013-C-04).

References

Chang, D, Yang FY, Yan JJ, Wu YQ, Bai SQ and Liang XZ 2012. SRAP analysis of genetic diversity of nine native populations of wild sugarcane, Saccharum spontaneum, from Sichuan, China. Gen. Mol. Res. **11**: 1245-1253.

Committee NP 2010. Pharmacopoeia of People's Republic of China, vol. 2. China medical science press, Beijing.

Kimura M and Crow JF 1964. The number of alleles that can be maintained in a finite population. Genetics **49**: 725.

Lewontin R 1972. Evolutionary Biology 6 Dobzhansky, T: 391-398.

Li G and Quiros CF 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction:its application to mapping and gene tagging in Brassica. Theor. Appl. Genet. **103**:455-461.

Liu S, Hu HL and Chen H 2013. Genomic DNA extraction from *Conyza blinii* Lévl. and the Uniform Design optimization of RAPD reaction system. CHIN TRADIT PAT MED **5**:1006-1010.

Ma J, Mao W, Zhu Y, Chen X and Bo T 2008. Establishment of DNA fingerprints on non-heading Chinese cabbage hybrids by RAPD,ISSR and SRAP. Paper presented at the Gardening Progress, Shanghai.

Manohar SH, Murthy HN and Ravishankar K 2013. Genetic diversity in a collection of Cucumis sativus L. assessed by RAPD and ISSR markers. J. Plant Biochem. Biotechnol. **22**: 241-244.

Nei M 1972. Genetic Distance between Populations. The American Naturalist. **106**: 283-292.

Nei M 1973. Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. USA **70**: 3321-3323

Peng L, Wang Y, Zhu H and Chen Q 2011. Fingerprint profile of active components for Artemisia selengensis Turcz by HPLC - PAD combined with chemometrics. Food Chem. **125**: 1064-1071.

Rohlf FJ 1998. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System Version 2.0 User Guide. New York.

Shao QS, Guo QS, Deng YM and Guo HP 2010. A comparative analysis of genetic diversity in medicinal *Chrysanthemum morifolium* based on morphology, ISSR and SRAP markers. Biochem. Syst. Ecol. **38**: 1160-1169.

Song Z, Li X, Wang H and Wang J 2009. Genetic diversity and population structure of Salvia miltiorrhiza Bge in China revealed by ISSR and SRAP. Genetica **138**: 241-249.

Su Yf, Chen L, Luo Y, Xin C, Min L and Guo DA 2007. Chemical constituents and their anti ulcerogenic studies on whole herb of *Conyza blinii* (I). Chin Tradit Herbal Drugs **38**: 332-334.

Wang Z, Wang JE, Wang XM and Gao HW 2012. Assessment of genetic diversity in *Galega officinalis* L. using ISSR and SRAP markers. Genet. Resour. Crop. Evol. **59**: 865-873.

Xu J, Li A, Wang X, Qi J, Zhang L, Zhang G, Su J and Tao A. 2013. Genetic diversity and phylogenetic relationship of kenaf (*Hibiscus cannabinus* l.) accessions evaluated by SRAP and ISSR. Biochem Syst. Ecol. **49**: 94-100.

Xu Y, Fang Y, Chen G and Su C 2004. Study on Development & Means of TCM Industry in Panxi Area. J. MT SCI **9**: 6.

Yang CR, He ZT, Li XC and Zheng QT 1989. Blinin, a neoclerodane diterpene from *Conyza blinii.* Phytochem. **28**: 2.

(Manuscript received on 10 August, 2015; revised on 6 October, 2015)